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RESEARCH ARTICLE

Comparative Analysis of Muscle Transcriptome between Pig Genotypes Identifies Genes and Regulatory Mechanisms Associated to Growth, Fatness and Metabolism



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Abstract

Iberian ham production includes both purebred (IB) and Duroc-crossbred (IBxDU) Iberian pigs, which show important differences in meat quality and production traits, such as muscle growth and fatness. This experiment was conducted to investigate gene expression differences, transcriptional regulation and genetic polymorphisms that could be associated with the observed phenotypic differences between IB and IBxDU pigs. Nine IB and 10 IBxDU pigs were slaughtered at birth. Morphometric measures and blood samples were obtained and samples from *Biceps femoris* muscle were employed for compositional and transcriptome analysis by RNA-Seq technology. Phenotypic differences were evident at this early age, including greater body size and weight in IBxDU and greater *Biceps femoris* intramuscular fat and plasma cholesterol content in IB newborns. We detected 149 differentially expressed genes between IB and IBxDU neonates ($p < 0.01$ and Fold-Change > 1.5). Several were related to adipose and muscle tissues development (*DLK1*, *FGF21* or *UBC*). The functional interpretation of the transcriptomic differences revealed enrichment of functions and pathways related to lipid metabolism in IB and to cellular and muscle growth in IBxDU pigs. Protein catabolism, cholesterol biosynthesis and immune system were functions enriched in both genotypes. We identified transcription factors potentially affecting the observed gene expression differences. Some of them have known functions on adipogenesis (*CEBPA*, *EGRs*), lipid metabolism (*PPARGC1B*) and myogenesis (*FOXOs*, *MEF2D*, *MYOD1*), which suggest a key role in the meat quality differences existing between IB and IBxDU hams. We also identified several polymorphisms showing differential segregation between IB and IBxDU pigs. Among them, non-synonymous variants were detected in

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several transcription factors as *PPARGC1B* and *TRIM63* genes, which could be associated to altered gene function. Taken together, these results provide information about candidate genes, metabolic pathways and genetic polymorphisms potentially involved in phenotypic differences between IB and IBxDU pigs associated to meat quality and production traits.

Introduction

The pig is the main species for meat consumption worldwide, 43% of total produced meat comes from pigs. Most production comes from the modern European pig breeds, which have been extensively selected and show optimized productivity and efficiency [1]. In the Mediterranean basin, there is also a significant production of unique high-quality traditional pork products from local breeds. The Mediterranean breeds, also known as fatty-pig breeds, have an ancient origin, and have been reared in extensive conditions for centuries, exposed therefore to harsh environments and seasonal variations in food availability (associated with the development of a thrifty genotype [2]). These breeds are smaller in size, have not undergone intense genetic selection and are less productive than modern breeds. As a consequence of the industrialization of pork production, three-quarters of the traditional breeds are extinct or marginalized [3]. The exception is the Iberian pig, the most representative Mediterranean traditional breed, which has an important commercial value based on high quality dry-cured products in terms of consumers' health and acceptance [4].

Peculiarities in Iberian pig metabolism drive its valued meat properties; Iberian pigs are characterized by higher fat deposition, fat desaturation and food intake [5, 6], as well as by higher circulating leptin levels in plasma [7] than lean pigs, suggesting a syndrome of leptin resistance. Moreover, the Iberian pig is also considered an amenable and robust biomedical model for obesity and associated cardiometabolic diseases since, when provided high levels of food, the animals are prone to the development of dyslipidemias, metabolic syndrome and type-2 diabetes [8]. On the other hand, as observed in other traditional breeds, productive performance is considerably lower than that of highly selected modern breeds. To improve reproductive and growth performances and primal cuts yield, in the last decades Duroc breed was introduced as terminal sire cross. Recently, Spanish law has accepted and regulated the use of Iberian X Duroc pigs to obtain "Iberian" products.

However, the introduction of Duroc genetics is associated with a decrease in meat quality, mainly determined by a decrease in intramuscular fat (IMF) and monounsaturated fatty acids (MUFA) contents [9]. Intramuscular fat content and fatty acid composition are the main factors affecting meat quality and are highly dependent on genetic type and diet [10]. Intramuscular fat content is determined both by number and size of adipocytes within muscle fibers. During prenatal development and immediately after birth, preadipocyte differentiation is a very active process that slow down with animal growth [11]. Later in growth adipocyte hypertrophy is the most important issue affecting IMF content, although hyperplasia is maintained in the adult animal to a lesser extent [12]. Thus, birth is a critical time-point to investigate the adipocyte differentiation process. On the other hand, IMF composition and fatty acids profile depend on lipogenesis and fatty acids metabolism. It has been reported that breed affects adipogenesis, lipogenesis and their timing, as well as the expression patterns of adipocyte differentiation-related genes [13]. In this sense, Iberian pig is considered a more precocious breed than Duroc pig [14].

Due to the influence of the genetic background on productive and meat quality traits, research in the past few decades has been focused on understanding the genetic basis of cell growth and development, myogenesis and metabolism [15]. Recently, new interest has arisen towards the understanding of genetic mechanisms underlying lipid synthesis and accumulation, due to its importance in meat quality [13]. Different approaches such as candidate gene expression studies or cDNA microarray analysis have been used to investigate genetic aspects of target parameters. Some studies based on the microarray technology investigated transcriptome differences among Iberian pig and Large White or Duroc pig in endocrine tissues [16] and between Iberian and Iberian X Duroc crossbred pigs in *Longissimus dorsi* muscle [14].

Currently, the availability of the RNA-Seq technology has allowed the assessment of global changes in transcriptome of a number of species including pigs [15], because of its greater accuracy and reproducibility than microarray technology [17, 18]. RNA-Seq allows measuring not only gene expression, but also examining genome structure identifying SNP and other structural variation such as indel and splice variants. Some applications of this technology include transcript quantification, allele-specific expression, novel transcript discovery or single nucleotide polymorphism (SNP) discovery [19]. In pigs, several RNA-Seq studies have been carried out for assessing differences in the transcriptome of muscle, fat, liver or hypothalamus among breeds or phenotypically extreme individuals within a breed for characters of interest [15, 20, 21]. The RNA-Seq technology is still scarcely applied to the Iberian breed, with studies comprehending the assessment of phenotypically extreme individuals for fatty acids composition [22] or the exploration of gonad transcriptome in Iberian and Large White pigs [23]. However, to the best of our knowledge, there are not RNA-Seq technology-based studies focused on genetic differences between Iberian and other breeds aimed at improving meat quality and productive traits.

Meat quality in Iberian pigs is of special interest for carcass cuts used in the dry curing industry such as the loin and the ham. A previous study assessed transcriptomic differences between pure Iberian and Duroc-crossbred Iberian pigs using microarray technology in the loin [14], but no information on ham muscles transcriptome exists. It is well known that different muscles differ in developmental timing, metabolic and physicochemical properties, including different responses to exercise [24]. *Biceps femoris* (BF) muscle is the biggest muscle in the ham and shows higher oxidative capacity, and lower drip loss than *Longissimus dorsi* muscle [25, 26]. Moreover, important differences exist regarding the IMF content of both muscles. Karlsson et al. (1993) reported higher IMF content in LD muscle in Yorkshire pig breed, whilst the opposite was reported for Iberian pigs, where BF showed remarkably greater IMF content than several others carcass muscles [27]. Also, transcriptomic and proteomic comparisons between muscles showed important functional differences, with 15–30% of proteome differing between LD and BF [24, 28]. On the other hand, transcriptomic studies performed sequentially along early development suggest the perinatal as a critical period to study genes affecting muscle and adipose cells growth and muscle fiber differentiation [20, 29], in agreement with the tissue differentiation timing commented previously. Moreover, environmental effects are minimized at this time point.

Hence, in agreement with previous considerations, the present study was carried out to study the BF muscle of newborn piglets in IB and in the IBxDU cross, aiming to: 1) Verify whether phenotypic differences are evident from the very early developmental stages (newborns) in these closely related populations; 2) Evaluate changes in gene expression in BF muscle that may be responsible for the observed phenotypic differences and identify pathways and networks in which those genes are involved; 3) Identify transcription factors affecting gene expression in order to establish potential new candidate genes affecting productive parameters and

meat quality; 4) Identify structural variants in these candidate genes, potentially involved in the observed expression differences.

These results are useful for the understanding of genetic pathways affecting pork production and may be also of translational value for the understanding of ethnic differences in obesity and associated disorders in lipid metabolism in human medicine.

Materials and Methods

Ethics statement

Animal manipulations were done in compliance with the regulations of the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 about the protection of animals used in research. The experiment was specifically assessed and approved (report CEEA 2010/003) by the INIA Committee of Ethics in Animal Research, which is the named Institutional Animal Care and Use Committee (IACUC) for the INIA.

Animals and sample collection

Ten pure Iberian sows raised in the same commercial farm were employed at their third gestation cycle. All females were managed in the same conditions. Five sows were mated to Iberian boars and five to Duroc boars. At birth, nine pure Iberian (IB) and 10 Iberian x Duroc (IBxDU) male piglets were randomly selected from the ten litters (two from each litter excepting one litter providing just one Iberian male). Blood samples were collected from newborns in sterile heparin blood vacuum tubes (Vacutainer Systems Europe, Meylan, France). Immediately after recovery, the blood was centrifuged at 1500g for 15 min and the plasma was separated and stored into polypropylene vials at -20°C until assayed for determination of glucose and lipids metabolism-indicating parameters. After blood collection, piglets were slaughtered. Several body development measures were obtained with a measure-tape: total body length (from the rostral edge of the snout to the tail insertion), ham length (from the anterior edge of the *Symphysis pubica* to the *articulatio tarsi*), total length of anterior and posterior limbs (from the distal edge of the hooves to the proximal edge of the *scapula* or *Symphysis pubica*, respectively) and thoracic, abdominal and ham circumferences. Carcasses were weighted and samples from BF muscle were vacuum-packed in low-oxygen permeable film and kept frozen at -20°C until fatty acid composition analysis. Prior to fatty acid analysis, muscle samples were freeze dried for two days in a lyophilizer (Lyoquest, Telstar, Tarrasa, Spain) and grounded in a Mixer Mill MM400 (Retsch technology, Haan, Germany) until muscle was completely powdered. For transcriptomic analysis, BF samples were immediately frozen in liquid nitrogen and maintained at -80°C until RNA extraction.

The metabolic status of the newborn piglets was evaluated. Glucose, fructosamine, triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c) plasmatic levels were measured with a clinical chemistry analyzer (Saturno 300 plus, Crony Instruments s. r. l., Rome, Italy).

Tissue composition analysis

Biceps femoris muscle IMF content was quantified using the method proposed by Segura and López-Bote [30] based on gravimetric determination of lipid content. Fatty acid methyl esters (FAMES) were identified by gas chromatography as described by López-Bote *et al* [31] using a Hewlett Packard HP-6890 (Avondale, PA, USA) gas chromatograph equipped with a flame ionization detector and a capillary column (HP-Innowax, 30 m \times 0.32 mm i.d. and 0.25 μm

polyethylene glycol-film thickness). Results were expressed as grams per 100 grams of detected FAMES.

Transcriptomic analysis

RNA extraction. A total of 12 animals were randomly selected to perform transcriptomic analysis, representing all available litters (6 animals of each genetic type). Total RNA was extracted from 50–100mg samples of *BF* muscle using the RiboPure™ of High Quality total RNA kit (Ambion, Austin, TX, USA) following the manufacturer's recommendations. RNA was quantified using a NanoDrop-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The quality of the RNA was evaluated using the RNA Integrity Number (RIN) value from the Agilent 2100 Bioanalyzer device (Agilent technologies, Santa Clara, CA, USA). The RIN values ranged from 7.5 to 9.8.

Library construction and RNA sequencing. Sequencing libraries were made using the mRNA-Seq sample preparation kit (Illumina Inc., Cat. # RS-100-0801) according to manufacturer's protocol. Each library was sequenced using TruSeq SBS Kit v3-HS, in paired end mode with the read length 2x76bp on a HiSeq2000 sequence analyzer (Illumina, Inc). Images from the instrument were processed using the manufacturer's software to generate FASTQ sequence files.

Mapping and assembly. Sequence reads were analyzed using CLC Bio Genomic workbench software 7.0 (CLC Bio, Aarhus, Denmark). Quality control analysis was performed using the NGS quality control tool, which assesses sequence quality indicators based on the FastQC-project (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Quality was measured taking into account sequence-read lengths and base-coverage, nucleotide contributions and base ambiguities, quality scores as emitted by the base caller and over-represented sequences [32]. All the samples analyzed passed all the QC parameters having the same length (76 bp), 100% coverage in all bases, 25% of A, T, G and C nucleotide contributions, 50% GC on base content and less than 0.1% over-represented sequences. A hierarchical clustering of the samples was also performed. One IBxDU pig was discarded for further analysis because the sample deviated largely from the expected grouping in the clustering analysis, probably due to RNA sampling or processing problems. Sequence paired-end reads (76bp) were assembled against the annotated Scrofa10.2 reference genome (<http://www.ncbi.nlm.nih.gov/genome/?term=sus+scrofa>) using the genome, annotated genes and mRNA tracks. Data was normalized by calculating the 'fragments per kilo base per million mapped reads' (FPKM) for each gene [33].

Differential expression analysis. The statistical analysis was performed using the total exon reads as expression values by the Empirical analysis of differential gene expression tool. This tool is based on the EdgeR Bioconductor package [34] and uses count data (i.e. total exon reads) for the statistical analysis. Genes were filtered according to two criteria: a minimum mean group expression greater than 0.5 FPKM in at least one group and a Fold-Change (FC) of the expression differences between IB and IBxDU groups equal or higher to 1.5. Finally, those genes with a $p \leq 0.01$, corresponding to a false discovery rate (FDR) value ≤ 0.23 , were considered as differentially expressed (DE).

Systems biology study. The biological interpretation of the DE genes observed in *BF* muscle was performed using three complementary approaches, in order to identify enriched GO terms, pathways and networks involving the DE genes, and potential regulators causing the observed changes in gene expression.

The enrichment analysis was carried out using the Wilcoxon test tool in the Consensus-PathDB database [35], available at the Max Plank Institute website, which provides batch

enrichment analyses to highlight the most relevant GO terms associated to a gene list. Both, the list of genes overexpressed in IB and IBxDU were used. Functional terms with p -values lower than 0.05 were considered enriched in the annotation categories. The p -values are corrected for multiple testing using the FDR method and are presented as q -values.

Additionally, Ingenuity Pathway Analysis, (IPA)(Ingenuity Systems, Qiagen, California) software was employed to identify and characterize biological functions, gene networks and canonical pathways affected by the DE genes.

Regulatory transcription factors (TRF), which could potentially affect the DE genes in the dataset were also studied by following complementary approaches. First, Regulatory Impact Factors (RIF1 and RIF2) metrics [36, 37] were calculated for the whole set of DE genes obtained conditional on genetic type (149 genes). RIF1 assigns an extreme score to those TRF that are consistently most differentially co-expressed with the highly abundant and highly DE genes; RIF2 assigns an extreme score to those TRF with the most altered ability to act as predictors of the abundance of DE genes. Candidate TRFs in pigs were obtained from Animal TFDB (<http://www.bioguo.org/AnimalTFDB/BrowseAllTF.php?spe=Susscrofa>). A total of 1038 TRF were retrieved. Among them, 723 showed expression values greater than 0.5 FPKM in at least one experimental group and thus, were used in the RIFs metrics approach.

The RIF1 and RIF2 values were computed for the i^{th} TRF as follows:

$$RIF1_i = \frac{1}{n_{de}} \sum_{j=1}^{j=n_{de}} \hat{a}_j \hat{d}_j (r1_{ij} - r2_{ij})^2 \text{ and}$$

$$RIF2_i = \frac{1}{n_{de}} \sum_{j=1}^{j=n_{de}} [(e1_j r1_{ij})^2 - (e2_j r2_{ij})^2]$$

where n_{de} is the number of DE genes, a_j and d_j the estimated average expression and differential expression of the j^{th} DE gene, $r1_{ij}$ and $r2_{ij}$ the co-expression correlation between the i^{th} TRF and the j^{th} DE gene in each one of the genetic types and being $e1_j$ and $e2_j$ the expression of the j^{th} gene in each genetic type [38]. Both RIF measures for each analyzed TRF were transformed to standardized z -scores by subtracting the mean and dividing by its standard deviation. We identified relevant TRF as those with extreme RIF z -scores according to the corresponding confidence intervals (CI) calculated by bootstrap. In each iteration of bootstrapping, a set of $n_{de} = 149$ genes were randomly selected from the 11392 expressed genes, and the RIF1 and RIF2 z -scores of the 723 TRF were calculated. The procedure was repeated 10,000 times for each scenario to obtain the corresponding 95 and 99% CI intervals of both z -scores. Complementarily, IPA software was employed to identify and characterize potential regulators using two different tools, the *upstream regulators* and the *regulators* tools. Both of them identify known regulators that may be affecting expression of the dataset of DE genes. IPA-identified regulators include genes, but also other molecules as drugs. Thus, out of the identified regulators, only genes that were also included in the RIFs metrics candidate TRF list (which consisted of 723 TRF) were considered (genes included in the animal TFDB and with expression values higher than 0.5 FPKM in at least one experimental group). Using the information obtained from the TRF study, an additional search for enriched pathways and networks was carried out with IPA software considering both, DE genes and TRF.

Structural variants analysis. A search of structural variants was performed by pooling the reads coming from all animals in each genetic type, and comparing the variants found in each group. The probabilistic variants detection tool (CLC Bio Genomic workbench) was used to perform the variant calling analysis. Single-end read alignments were not ignored. The minimum coverage in a locus to be considered was set up as 10 and the variant probability as 90.

The variant probability parameter defines how good the evidence has to be at a particular site for the tool to report a variant at that location. Specifically, the variant probability threshold set as 90 means that any candidate variation in the genome must have a probability lower than 0.1 (1–0.9) of being the same as the reference sequence, to be considered as a variant. Variants with an allele frequency under 5% and/or coverage under 30 reads for the general variant analysis and under 10 reads in the candidate genes variant analysis were not considered. Variants were considered to be potentially fixed (frequency greater or equal to 90%) or segregating (frequency lower than 90%).

The variants identified in genes corresponding to transcription factors (i.e. *EGR2*, *FOS*, *FOXO1*, *FOXO3*, *IRF1*, *STAT5B*, *HOXA9*, *ATF4*, *TP53*, *NOR-1*, *ABRA*, *ATF3* and *PPARGC1B*) were functionally evaluated using the variant effect predictor (VEP) tool from Ensembl (www.ensembl.org/info/docs/tools/vep/) which includes information about amino acid change localization and consequences, affected transcripts, and SIFT [39] and PolyPhen [40] scores.

Results validation by RT qPCR

RNA obtained from the 11 animals under study was employed to perform the technical validation of the differential expression of some genes that were either upregulated in IB, upregulated in IBxDU or not DE between genetic types. This technical validation was performed by studying the Pearson correlation between the expression values obtained from RNA-Seq data (FPKM) and the normalized gene expression data obtained by RT qPCR. Moreover, RNA obtained from all the available animals (9 IB and 10 IBxDU) was used to quantify expression differences of such genes.

First-strand cDNA synthesis was carried out with Superscript II (Invitrogen, Life Technologies, Paisley, UK) and random hexamers in a total volume of 20 µl containing 1 µg of total RNA and following the supplier's instructions.

The expression of 9 genes was quantified by qPCR. Primer pairs used for quantification were designed using Primer Select software (DNASTAR, Wisconsin, USA) from the available GENBANK and/or ENSEMBL sequences, covering different exons in order to assure the amplification of the cDNA. Sequence of primers and amplicon lengths are indicated in [S1 Table](#). Standard PCRs on cDNA were carried out to verify amplicon sizes. Quantification was performed using SYBR Green mix (Roche, Basel, Switzerland) in a LightCycler480 (Roche, Basel, Switzerland), following standard procedures. Data were analyzed with LightCycler480 SW1.5 software (Roche, Basel, Switzerland). All samples were run in triplicate and dissociation curves were carried out for each individual replicate. Single peaks in the dissociation curves confirmed the specific amplification of the genes. For each gene, PCR efficiency was estimated by standard curve calculation using four points of cDNA serial dilutions. Mean Cp values were employed for the statistical analyses of differential expression. Stability of four endogenous genes (i. e. *GAPDH*, *B2M*, *TBP* and *ACTB*) was calculated using Genorm software [41]. The *TBP* and *ACTB* genes were selected as the most stable endogenous genes to normalize the data. The qPCR expression data normalization was performed using normalization factors calculated with Genorm software. Relative quantities were divided by the normalization factors, which were the geometric means of the two reference genes quantities.

Statistical analyses of tissue composition and qPCR expression quantification

Phenotypic data were analyzed as a completely randomized design using the general linear model (GLM) procedure using SAS version 9.2 (SAS Inst. Inc., Cary, NC; 2009). The mean and genetic type were considered as systematic effects, and residual effects as random. Carcass

weight was used as covariate when it was significant and removed from the model when it was insignificant. The animal was the experimental unit for all analysis. The results were considered to be significant at $p\text{-value} < 0.05$.

Statistical analysis of gene expression data was carried out following the method proposed by Steibel *et al* [42] which consists of the analysis of cycles to threshold values (C_p), for the target and endogenous genes using a linear mixed model. The following model was used for analyzing the joint expression of the target and control genes in different tissues:

$$y_{gikr} = TG_{gi} + B_{gk} + D_{ik} + e_{gikr}$$

where, $y_{gikr} = -\log_2(E_g^{-C_{p_{gikr}}})$, E_g is the efficiency of the PCR of gth gene, $C_{p_{gikr}}$ is the value obtained from the thermocycler software for the gth gene from the r th replicate in a sample collected from the k th animal of the i th genetic type, TG_{gi} is the specific effect of the i th genetic type on the expression of gene gth, B_{gk} is specific random effect of the k th pig on the expression of gene gth, D_{ijk} is a random sample-specific effect common to all the genes, and e_{gikr} is a residual effect.

To test differences in the expression rate of genes of interest ($diff_{TG}$) between classes normalized by the endogenous genes, different contrasts were performed between the respective estimates of TG levels. Significance of $diff_{TG}$ estimates was determined with the t statistic. To obtain FC values from the estimated $diff_{TG}$ values, the following equation was applied:

$$FC = 2^{-diff_{TG}}$$

$P\text{-values} < 0.05$ were considered statistically significant.

To validate the global RNA-Seq results, the concordance correlation coefficient (CCC) [43] was calculated between the FC values estimated in BF muscle from RNA-Seq and qPCR expression measures for the 9 genes analyzed by the two technologies (RNA-Seq and qPCR).

Results and Discussion

Phenotypic differences between genetic types

The results obtained in the present study constitute the first assessment of phenotypic differences between IB and IBxDU piglets at birth. There are several studies evaluating phenotypic differences between both genotypes at weaning or adulthood [9, 14, 44–46]. Pure Iberian and crossbred piglets were slaughtered at birth at an average of 1.2 and 1.8 kg live weight, respectively (SEM = 0.06). Genetic type affected all the carcass phenotypic parameters: IBxDU neonates were bigger and heavier ($p < 0.001$) than IB newborns (Table 1), reflecting previously reported differences in the same traits in adult animals [45, 46]. Birth is an interesting sampling time to study differences in metabolism and growth, in which environmental influences are minimized, but maternal effects may have to be considered. Unfortunately no information regarding the employed sows' phenotype was available, but all of them originated from the same commercial Iberian population and were checked for breed purity by genotyping several markers usually employed for breed origin authentication, showing homocycosity for Iberian alleles. Moreover age and productive cycle were also homogeneous among them as indicated in the methods section, and thus, we don't expect that potential sow effects may interfere with the observed between-genotype differences regarding piglet's phenotype or transcriptome.

The assessment of differences in glucose and lipids metabolism (Table 1) showed that purebred IB piglets have greater plasma levels of total and HDL cholesterol, and triglyceride than IBxDU neonates. These differences at birth are concordant with the similar differences previously found between purebred Iberian and lean crossbred (Large White x Landrace x Pietrain) fetuses [47]. Cholesterol is of vital importance for the offspring as a key constituent of cell

Table 1. Carcass, *Biceps Femoris* and metabolism phenotypic characteristics in IB and IBxDU piglets.

	Genetic type		SEM ³	p-value
	IBxDU ¹	IB ²		
Carcass characteristics				
Carcass weight, kg	1.41	0.96	0.05	0.0005
Ham weight, kg	0.16	0.11	0.00	0.0008
Total body lenght, cm	40.20	35.50	0.31	0.0004
Ham lenght, cm	7.45	6.33	0.12	0.0009
Forelimb lenght, cm	12.35	10.83	0.12	0.0042
Hind limb lenght, cm	15.95	13.83	0.12	0.0016
Torax circumference, cm	25.15	22.06	0.14	0.0010
Abdomen circumference, cm	18.90	17.28	0.19	0.0486
Ham circumference, cm	12.55	10.89	0.15	0.0020
Lipid and glucose metabolism-related plasma indicators				
Cholesterol, mg/dl	62.19	102.36	5.60	0.0030
Fructosamine, mg/dl	169.70	133.67	10.37	0.1009
Glucose, mg/dl	132.40	123.44	10.80	0.6839
LDL ⁴ , mg/dl	42.16	45.82	4.40	0.4496
HDL ⁵ , mg/dl	22.38	41.20	4.25	0.0176
Triglycerides, mg/dl	30.00	76.67	5.11	0.0003
<i>Biceps femoris</i> muscle fatty acids composition (g/100 g total fatty acids)				
IMF ⁶ , %	1.72	2.21	0.09	0.0142
C12:0	0.65	0.58	0.03	0.2321
C14:0	2.57	2.32	0.12	0.3189
C15:1	1.28	1.18	0.06	0.3762
C16:0	25.90	25.44	0.19	0.2379
C16:1 n-9	1.90	2.09	0.05	0.3854
C16:1 n-7	5.38	4.57	0.21	0.0773
C17:0	1.69	1.44	0.07	0.0814
C17:1	0.91	0.81	0.06	0.3858
C18:0	10.85	9.96	0.25	0.1014
C18:1 n-9	23.80	25.82	0.56	0.0921
C18:1 n-7	6.15	5.69	0.18	0.2163
C18:2 n-6	7.31	9.17	0.60	0.1395
C20:1 n-9	0.53	0.53	0.01	0.9701
C20:2 n-6	0.41	0.40	0.03	0.8331
C20:3 n-6	0.62	0.55	0.02	0.0293
C20:4 n-6	6.31	5.99	0.20	0.4264
C22:1 n-9	1.21	1.08	0.05	0.1956
C22:4 n-6	1.58	1.27	0.09	0.0925
C22:5 n-3	0.48	0.50	0.02	0.5405
C22:6 n-3	0.67	0.62	0.02	0.1959
ΣSFA ⁷	41.66	39.74	0.42	0.0350
ΣMUFA ⁸	41.01	41.77	0.40	0.3530
ΣPUFA ⁹	17.34	18.49	0.50	0.2639
UI ¹⁰	96.20	97.79	0.90	0.3904
Σn-3 ¹¹	1.77	1.67	0.04	0.1940
Σn-6 ¹²	15.56	16.82	0.48	0.2089
Σn-6/Σn-3	8.78	10.17	0.30	0.0319

(Continued)

Table 1. (Continued)

	Genetic type		SEM ³	p-value
	IBxDU ¹	IB ²		
Σ MUFA/ Σ SFA	0.99	1.05	0.02	0.0659

¹ IBxDU = Iberian x Duroc crossbred pigs (n = 10)

² IB = Purebred Iberian pigs (n = 9)

³SEM = Standard error of the mean

⁴LDL = Low density lipoproteins

⁵HDL = High density lipoproteins

⁶IMF = Intramuscular fat

⁷ Σ SFA = Sum of saturated fatty acids

⁸ Σ MUFA = Sum of monounsaturated fatty acids

⁹ Σ PUFA = Sum of polyunsaturated fatty acids

¹⁰UI = Unsaturation index = $1 \times (\% \text{ monoenoics}) + 2 \times (\% \text{ dienoics}) + 3 \times (\% \text{ trienoics}) + 4 \times (\% \text{ tetraenoics}) + 5 \times (\% \text{ pentaenoics}) + 6 \times (\% \text{ hexaenoics})$ [126]

¹¹ Σ n3 = Sum of n-3 fatty acids

¹² Σ n6 = Sum of n-6 fatty acids

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membranes and the precursor of hormones and metabolic regulators [48, 49]. Placental and fetal tissues have the capacity for *de novo* cholesterol synthesis [50] but the high demand from the fetuses makes the transport of maternal cholesterol through the placenta necessary [51, 52]. Triglycerides are also indispensable as a major source of energy for the developing fetus and are also transferred from maternal circulation [53, 54]. Previous studies have found that adequate availability of cholesterol and triglycerides is even more critical in fatty-pigs breeds [55], which have higher values of plasma lipids indexes than lean breeds [47].

These results reinforce that genetic differences between fatty-pigs and lean breeds are established from prenatal stages and, together with previous results, may also give evidence of a genetic predisposition for lipid metabolism alterations in the Iberian breed. The same findings regarding plasma cholesterol and triglycerides levels have been reported in humans with familial combined hyperlipidemia, the most common genetic form of hyperlipidemia in human [56, 57] and in the Rapacz familial hypercholesterolaemic swine model.

Regarding the IMF content and composition in BF (Table 1), IB showed almost 30% higher IMF content than IBxDU piglets ($p = 0.014$). The genetic type affected IMF composition as well, IB pigs showing greater Σ n-6/ Σ n-3 ratio ($p = 0.031$) (mainly due to greater proportion of C18:2 n-6), and lower Σ SFA (saturated fatty acids) content ($p = 0.035$). Also, a trend for a higher oleic acid content was observed in IB pigs ($p = 0.092$). As reported in previous studies, crossing with Duroc sires decreased IMF concentration, in agreement with differences observed in adult pigs [9, 14].

The differences observed between IB and IBxDU in parameters such as body size and weight, lipid metabolism-related indicators or IMF were surprising taking in account the early stage of development. This highlights the importance of improving the knowledge on molecular aspects responsible for such phenotypic differences at very early ages with the dual purpose of improving production in local breeds with distinctive products and providing adequate models for human diseases.

Identification of differentially expressed genes by RNA-Seq analysis

An average of approximately 79 million sequence reads was obtained for each individual sample; these were assembled and mapped to the annotated Sscrofa10.2 genome assembly (22,861 genes). In all samples, 67–77% of the reads were categorized as mapped reads to the porcine reference sequence.

The FPKM values were used to establish the total number of genes expressed in muscle transcriptome (>0.5 FPKM). Approximately 50% of total porcine annotated genes in the Sscrofa10.2 genome assembly were expressed in the studied samples (an average of 11,392 genes out of 22,861 annotated genes).

Ninety-five genes were overexpressed in IB (FC ranging from 1.9 to 12) and 54 genes were overexpressed in IBxDU (FC ranging from 2 to 27.2) ($p < 0.01$) (S2 Table).

Large expression differences were observed for the genes *MARCO* (27.2x) and *CXCL13* (27.1x), which showed greater expression level in IBxDU than in IB piglets. *MARCO* and *CXCL13* genes are both related to immune response. *MARCO* is also involved in cytoskeleton and cell morphology determination of certain immune cells [58] and *CXCL13* has also been found to be upregulated in adipocytes when compared to preadipocytes [59].

On the other hand, another unidentified protein (ENSSSCG00000023287; FC = 12.5x), ortholog of human *MYL6B* gene, and the pig genes *CIART* (8.4x) and *ATF3* (7.8x) were upregulated in IB piglets at birth. *CIART* is a transcription repressor of the mammalian circadian clock that inhibits the activators *CLOCK* and *BMAL-1* [60]. The mammalian circadian clock regulates sleep–wake rhythms, body temperature, blood pressure, hormone production, immune system or cell cycle (reviewed in [61]). It is also important for energy homeostasis regulation, as multiple genes involved in nutrient metabolism and metabolically related hormones such as insulin or leptin display rhythmic oscillations [62]. It has been reported that animals deficient in *BMAL-1* show altered lipid homeostasis (i.e. an increase in the levels of circulating fatty acids, including triglycerides, free fatty acids, and cholesterol) and metabolic syndrome [63], which is in agreement with the phenotypic results observed in IB pigs. *ATF3* codes for a transcription factor considered as an adaptation-response gene involved in a variety of processes such as immunity, regulation of the cell cycle and apoptosis [64], and cellular stress response [65].

Some other interesting DE genes are related to muscle and adipose tissue development, for example *SLC2A4*, *FGF21*, *UBC*, or *ACHE* [66–69]. Moreover, three DE genes (*DLK1*, *MYH10* and *ZWILCH*) were also observed to be DE between IB and IBxDU in a previous study [14], where *Longissimus dorsi* transcriptome was compared at weaning. *DLK1* is a transmembrane protein expressed in preadipocytes but not in mature adipocytes [70], thus, greater expression in IB neonates (2.6x) suggests greater number of undifferentiated preadipocytes in IB than in IBxDU piglets, possibly associated with a greater adipogenic potential. However, in a previous study, *DLK1* gene was found to be upregulated in IBxDU pigs at weaning [14]. The different age of sampling could explain the opposite results: IB piglets may be born with higher amounts of preadipocytes that differentiate faster than those from IBxDU pigs thus, leading to lower preadipocyte content at weaning age. Similarly, a different pattern for myocyte differentiation has been reported between high and low muscle growth efficiency breeds, such as Landrace, Lantang, Pietrain or Duroc [29, 71]. In Landrace, myocyte differentiation develops faster than in low efficiency breeds [29, 71]. In addition, the proliferation and differentiation of preadipocytes are stronger and faster in Bamei than in Landrace (representing a fat- and lean-type pig breed, respectively) [17, 72]. Thus, we suggest a faster adipocyte differentiation in IB pigs at an early age that may conclude earlier than in IBxDU pigs.

MYH10 gene codes for a heavy-chain myosin, and was also found upregulated in IBxDU pigs when compared to IB pigs at birth and at weaning [14], which suggests a greater development of muscular cells in crossbreds. The gene *ZWILCH* is overexpressed in IB at both ages; it is involved in cell proliferation and differentiation, and it may also play a role in the control of adipogenesis [73], thus being an interesting candidate to explain phenotypic differences in adipogenesis and lipogenesis.

In order to validate the results obtained from the RNA-Seq analysis, the relative expression of some DE genes (upregulated in both genetic types) and a few non-DE genes was assessed by qPCR in all the available samples. Five out of the seven genes identified as DE by RNA-Seq technology were validated by qPCR. The remaining two genes showed a trend ($p = 0.085$) and a numerical difference ($p = 0.105$). Non-DE genes showed similar results using both technologies (S3 Table). A concordance correlation coefficient, used to assess technical validation in high throughput transcriptomic studies [43] was calculated ($CCC = 0.93$) and denoted a high general concordance between RNA-Seq and qPCR expression values. In general good individual correlation values were obtained (S3 Table). Fold-Change and significance tended to be greater when expression differences were analyzed by RNA-Seq technology, in accordance with its higher sensitivity [74].

Biological interpretation of the differential expression results

Different approaches were used to perform an exhaustive and robust biological interpretation of the results obtained in the transcriptome study. Results obtained from the Gene Ontology (GO) term overrepresentation analysis, performed to detect active biological processes differing in both IB and IBxDU, are shown in Table 2. In addition, IPA software was used to find biological functions overrepresented in both genetic types (S4 Table and Figs 1–3) and to identify pathways (Table 3) and networks (Fig 4) associated with the DE genes.

Upregulated functions and pathways in *Biceps femoris* muscle from purebred Iberian piglets. Enriched biological functions in IB piglets identified by IPA software were mainly related with lipid and glucose metabolism (i.e. *Concentration of lipid*, *Synthesis of lipid*, *Insulin resistance*, *Quantity of adipose tissue* or *Fatty acid metabolism*; Fig 1) and with muscle growth (S4 Table). In agreement, several DE genes with known functions related to lipid metabolism such as *FOS*, *FDFT1*, *SLC4A2*, *TRIM63*, *EPXH1*, *ALOX12B*, *FOXO3A* or *ACHE* were overexpressed in IB, possibly associated to the higher amount of adipose tissue observed in BF muscle of IB when compared to IBxDU pigs (Table 1). Similar results have been found in IB and IBxDU piglets at 28 days of age in loin muscle [14]. However, in the previous study, a different set of DE genes related to lipid metabolism was found, probably due to the high complexity of processes and molecular mechanisms regulating lipid metabolism at that stage of development. In another study comparing the muscle transcriptome of two divergent breeds for muscle and fat deposition, several muscle metabolism-related genes were identified as potential regulators of IMF deposition [29], such as *FOS* and *ABRA* genes, identified also in the present study.

Accordingly, several pathways enriched in IB pigs ($p < 0.05$) (Table 3) were related to the control of energy homeostasis (*Wnt/Ca+*, *Glutamine Biosynthesis* or *Fatty Acid α -oxidation* pathways) and to protein synthesis and cell growth (*Growth Hormone (GH) Signaling* and *IGF-1 Signaling*); IGF-1 is essential during prenatal and GH during postnatal growth [75]. The effect of GH and IGF-1 on adipose tissue development and metabolism is controversial, as both have been proposed to play either a positive or a negative role on adipocyte differentiation [76, 77]. Thus, in IB newborns, the activation of these pathways might be associated to both muscle and preadipocytes development and differentiation. Accordingly, the *adipogenesis* pathway showed a trend for enrichment ($p = 0.055$).

Table 2. Gene Ontology (GO) overrepresented terms regarding the biological process category.

	*GO term	p-value	q-value	Term name
COMMON	GO:0007165	9.54E-07	6.96E-05	Signal transduction
	GO:0031323	3.81E-06	1.39E-04	Regulation of cellular metabolic process
	GO:0009059	6.10E-05	6.37E-04	Macromolecule biosynthetic process
	GO:0044267	1.22E-04	8.10E-04	Cellular protein metabolic process
	GO:0010646	2.44E-04	1.11E-03	Regulation of cell communication
	GO:0030154	2.44E-04	1.11E-03	Cell differentiation
	GO:0048584	7.81E-03	1.84E-02	Positive regulation of response to stimulus
IB ¹	GO:0010467	1.22E-04	8.10E-04	Gene expression
	GO:0043412	1.22E-04	8.10E-04	Macromolecule modification
	GO:0036211	2.44E-04	1.11E-03	Protein modification process
	GO:0009889	4.88E-04	1.98E-03	Regulation of biosynthetic process
	GO:0090304	9.77E-04	3.56E-03	Nucleic acid metabolic process
	GO:0019438	1.95E-03	5.48E-03	Aromatic compound biosynthetic process
	GO:0016070	1.95E-03	5.48E-03	RNA metabolic process
IBxDU ²	GO:0023056	7.81E-03	1.84E-02	Positive regulation of signaling
	GO:0002684	1.95E-03	1.86E-02	Positive regulation of immune system process
	GO:0050790	7.81E-03	3.49E-02	Regulation of catalytic activity
	GO:0016337	7.81E-03	3.49E-02	Single organismal cell-cell adhesion

¹ IB = Purebred Iberian pigs² IBxDU = Iberian X Duroc crossbred pigs

*GO terms are considered either common, when they are enriched in both genetic types and specific when the GO term is only enriched in one of the two genetic types.

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Upregulated functions and pathways in *Biceps femoris* muscle from Duroc-crossbred Iberian piglets. Enriched functions in IBxDU newborns ([S4 Table](#)) were related to cell growth and differentiation, such as *Recruitment and transmigration of cells*, *Hyperplasia or Transformation of fibroblast cell lines* ([Fig 2](#)), and to protein and muscle organization and accretion such as *Mass of organism*, *Quantity of protein in blood* and *Organization of cytoskeleton* ([Fig 3](#)). Consistently, DE genes in IBxDU pigs clustered in 3 gene networks related to *Cell-To-Cell Signaling and Interaction*, *Hematological System Development and Function*, *Immune Cell Trafficking*, *Post-Translational Modification*, *Protein Folding*, *Cellular Assembly and Organization*, *Cellular Movement*, *Cell Signaling* and *Cellular Function and Maintenance*.

Some IBxDU upregulated genes involved in those functions were chemokines (i.e. *CCL19*, *CCL2* or *CXCL13*) and chemokine receptors (*ACKR1*), which have been reported to be involved in the process of myogenesis and muscle regeneration [78]. In our study, chemokines were associated with functions such as transmigration and recruitment of cells and organization of cytoskeleton (Figs 2 and 3), which could be related to muscle cell growth. Moreover, *MYH10*, a non-muscle myosin that regulates actin cytoskeleton remodeling, was found overexpressed in IBxDU and involved in cytoskeleton reorganization, a critical process during myogenesis [79]. Consistently, the *Actin Cytoskeleton Signaling* pathway was also enriched in IBxDU pigs in both the present study and at 28 day of age [14].

Mass of organism, associated with the overexpression of *PRSS8*, was predicted by IPA software to be enriched in IBxDU piglets, in accordance with the phenotypic differences in body weight and size found between pure and crossbred pigs. In agreement, a decrease in body weight was reported in mutant rats for gene *PRSS8* [80].

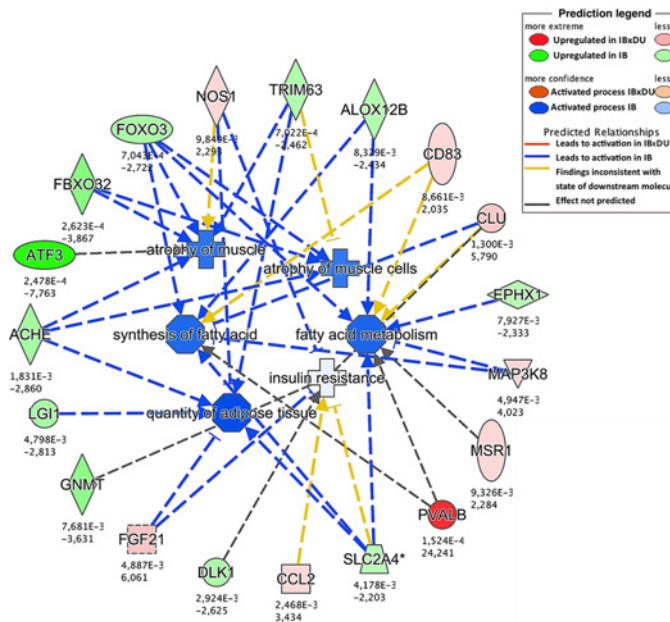


Fig 1. Enriched biological functions in Iberian (IB) pigs. The network generated by IPA software shows enriched biological functions in IB pigs (blue color) related to lipid metabolism and muscle atrophy. Lipid synthesis is upregulated in IB pigs. Muscle and protein degradation are also enriched, probably leading to a decreased muscle growth in those pigs. The network includes several transcription factors which may play key roles in the functional differences between genetic types, in agreement with the regulators study (*FOXO3*, *ATF3*, *TRIM63*).

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The enrichment of protein and muscle development-related pathways ($p < 0.05$), such as *Citrulline-Nitric Oxide Cycle*, *Superpathway of Citrulline Metabolism* or *nNOS Signaling in Skeletal Muscle*, support the greater muscle development in IBxDU piglets. Citrulline is a non-essential amino acid that plays a role in muscle development and affects body composition in

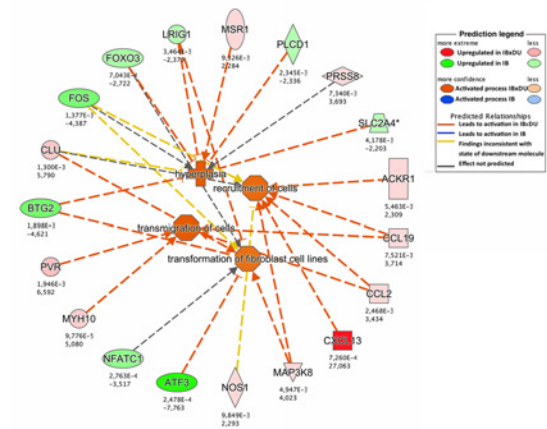


Fig 2. Enriched biological functions related to cell growth in crossbred (IBxDU) pigs. The network generated by IPA software shows enriched biological functions in IBxDU pigs (orange color) involved in cellular growth and differentiation. Upregulation of several chemokines as well as a myosin in IBxDU pigs, and downregulation of some transcription factors (*FOXO3*, *ATF3*) are responsible for the enrichment of such functions in crossbreds.

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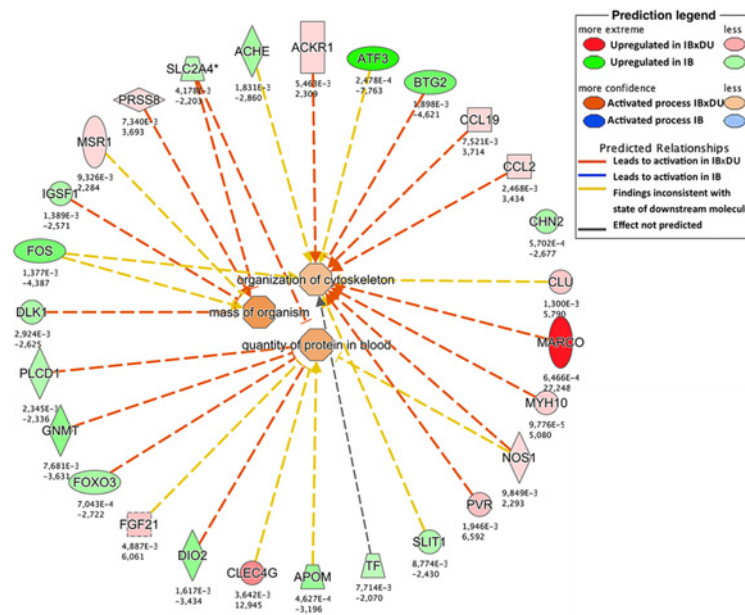


Fig 3. Enriched biological functions potentially related to muscle growth in crossbred (IBxDU) pigs. The network generated by IPA software shows enriched biological functions in IBxDU pigs (orange color) potentially related to muscle growth and body size, functions regulated by a wide variety of genes, from growth factors (*FGF21*) to immune system related genes (*MARCO*, *MSR1*).

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rats, increasing lean and decreasing adipose tissue when it is provided in the diet [81]. The *nNOS Signaling in Skeletal Muscle Cells* modifies the blood flow to the muscle [82]. Thus, the activation of these pathways suggests a greater nutrient input to muscle tissues in those pigs.

Upregulated functions and pathways in *Biceps femoris* muscle from both Duroc-crossbred and purebred Iberian piglets. Development-related processes depend on the genetic background, but also on the growth stage. At birth, when growth is a very active process, several functions and pathways enriched in both genetic types were observed. However, these common processes ended up in different phenotypic consequences. This might be because developmental mechanisms such as muscle growth or immune system development are tightly regulated and the final output depends on both the balance of activating and inhibiting pathways and the expression levels of genes involved in such processes.

Regarding cellular growth, the GO terms enrichment analysis retrieved several GO terms related to normal cell cycle and growth, such as cell differentiation, cellular protein metabolic process or macromolecule biosynthetic process that were common between both genetic types, supporting the importance of these processes in the early development of pigs. Accordingly, in 3 months-old Casertana pig (an autochthonous Italian fatty pig breed), GO terms close to the aforementioned were upregulated when compared to 6 months old pigs [83].

The importance of protein metabolism at birth is supported by the activation of the protein ubiquitination process in both genetic types. The *Protein Ubiquitination Pathway* was enriched in crossbred piglets ($p = 0.005$; Table 3), where mainly members of the family of Heat Shock Proteins (*HSPH1*, *HSPA4L* and *DNAJA1*) were found upregulated in IBxDU pigs. *DNAJA1*, together with *HSP27* have been reported to play a role in both cellular stress and meat quality; their expression was found to decrease IMF content [84], consistently with the results observed in IBxDU pigs. However, no enrichment in atrophy or degradation of muscle-related functions was observed in IBxDU piglets. Conversely, IB muscle showed activation of functions related

Table 3. Pathways significantly enriched in Purebred (IB) and Duroc-crossbred (IBxDU) Iberian pigs.

IB ¹		IBxDU ²	
Pathway	p-value	Pathway	p-value
PI3K Signaling in B Lymphocytes	<0.001	Agranulocyte Adhesion and Diapedesis	<0.001
NRF2-mediated Oxidative Stress Response	0.0022	LXR/RXR Activation	<0.001
Glutamine Biosynthesis I	0.0031	Atherosclerosis Signaling	<0.001
IL-17A Signaling in Fibroblasts	0.0051	Aldosterone Signaling in Epithelial Cells	0.0012
April Mediated Signaling	0.0060	Granulocyte Adhesion and Diapedesis	0.0019
LXR/RXR Activation	0.0060	TREM1 Signaling	0.0050
Epoxysqualene Biosynthesis	0.0062	Protein Ubiquitination Pathway	0.0054
B Cell Activating Factor Signaling	0.0066	Citrulline-Nitric Oxide Cycle	0.0071
Thyronamine and Iodothyronamine Metabolism	0.0091	IL-12 Signaling and Production in Macrophages	0.0155
Thyroid Hormone Metabolism I (via Deiodination)	0.0091	Superpathway of Citrulline Metabolism	0.0195
Wnt/Ca+ pathway	0.0129	nNOS Signaling in Skeletal Muscle Cells	0.0209
Tight Junction Signaling	0.0145	Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F	0.0251
AcutePhase Response Signaling	0.0151	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	0.0263
ERK5 Signaling	0.0158	Clathrin-mediated Endocytosis Signaling	0.0275
Growth Hormone Signaling	0.0191	Actin Cytoskeleton Signaling	0.0372
Clathrin-mediated Endocytosis Signaling	0.0191	Role of p14/p19ARF in Tumor Suppression	0.0417
Melatonin Signaling	0.0195	IL-17A Signaling in Fibroblasts	0.0468
Toll-like Receptor Signaling	0.0214		
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	0.0245		
UVA-Induced MAPK Signaling	0.0295		
Antioxidant Action of Vitamin C	0.0355		
IGF-1 Signaling	0.0355		
T Cell Receptor Signaling	0.0355		
Role of IL-17A in Psoriasis	0.0389		
Cholesterol Biosynthesis I	0.0389		
Cholesterol Biosynthesis II (24, 25-dihydrolanosterol)	0.0389		
Cholesterol Biosynthesis III (via Desmosterol)	0.0389		
Vitamin-C Transport	0.0417		
Glucocorticoid Receptor Signaling	0.0457		
FattyAcid α -oxidation	0.0479		

¹ IB = Purebred Iberian pigs

² IBxDU = Iberian X Duroc crossbred pigs

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to muscle atrophy (Fig 1), mainly driven by genes that stimulate muscle atrophy by means of the ubiquitin proteasome system (UPS) [85]. This system was also upregulated in IB when compared to IBxDU pigs at 28 days of age [14]; and in Basque (a French pig breed with low lean meat and high fat contents) when compared to Large White pigs [86]. Supporting the evidence of an activated protein ubiquitination process, the gene coding for Ubiquitin C (*UBC*) and *FBXO32* (one of the three ligase enzymes, together with *TRIM63* of the UPS) were also overexpressed in IB pigs.

In accordance with these results, genes implicated in these processes were found in the most significant gene network involving DE genes in both genetic types. This network was associated

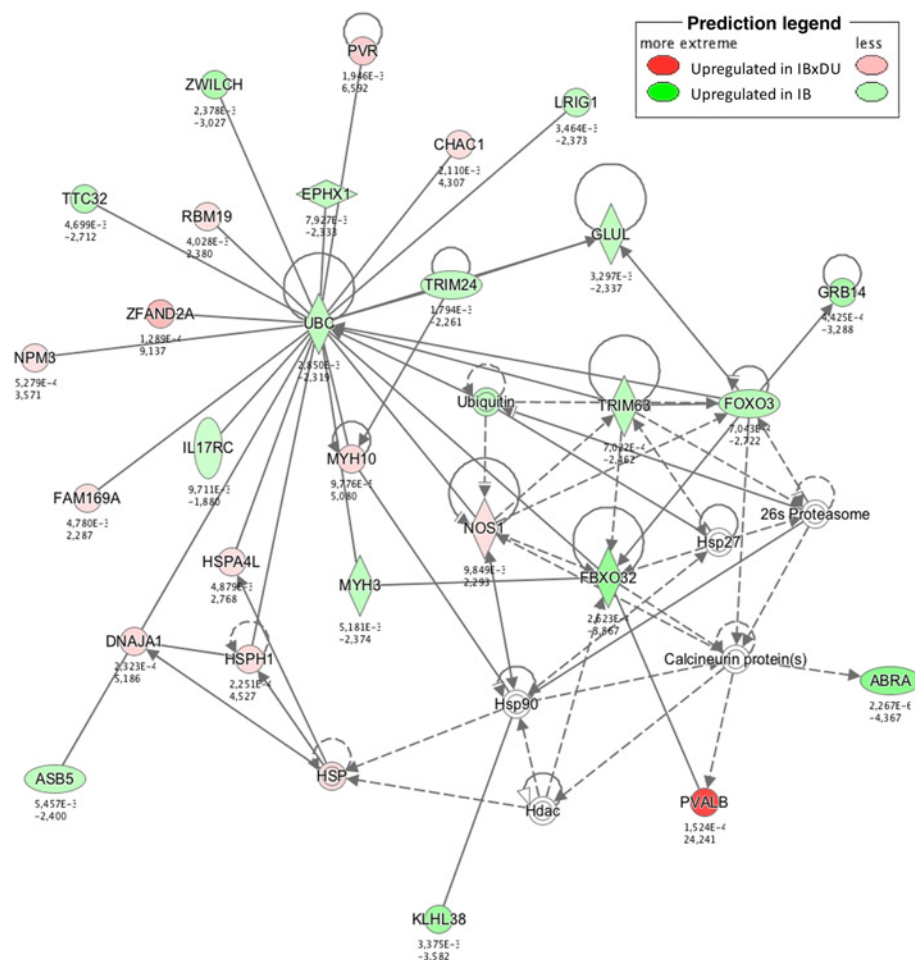


Fig 4. Gene network containing DE genes related to Cellular Compromise, Organismal Injury and Abnormalities and Skeletal and Muscular Disorders. This gene network shows several genes functionally interconnected and closely associated with the ubiquitin C (UBC), a central molecule in the network responsible for protein degradation. These genes are differentially expressed between genotypes and indicate the importance of the ubiquitination process in muscle growth of newborn pigs.

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with *Skeletal and Muscular Disorders*, as well as with *Cellular Compromise, Organismal Injury and Abnormalities* (Fig 4) and showed several genes related to protein catabolism (UBC, HSP, TRIM63 or FOXO3) among the most central genes in the network, which supports the importance of these genes in protein catabolism, a very active process in developing IBxDU and especially in IB pigs.

Iberian pigs show low protein accretion potential, although higher relative protein synthesis rate has been reported in IB than in lean Landrace pigs [87]. Since IB muscles are smaller than Landrace ones, it was suggested that a higher protein turnover rate in IB pigs should be responsible for the differences between protein synthesis and deposition [87]. In this context, it seems evident that this increased protein turnover in IB pigs is related to the activated muscle atrophy-related genes found in this study. Moreover, Damon et al. (2012) suggested that the activation of the UPS might affect meat tenderness, since proteasome would be one of the main endogenous proteolytic systems contributing post-mortem meat tenderization.

The UPS is also interconnected to the unfolded protein response (UPR), activated under endoplasmic reticulum stress situations. The UPR is involved in degradation of un and misfolded proteins and recently associated to the control of adipogenesis under situations of endoplasmic reticulum stress [88, 89]. It is noteworthy that *ATF3*, a transcription factor inducible by endoplasmic reticulum stress [65] was upregulated in IB pigs. This could suggest certain level of endoplasmic reticulum stress in IB pigs that might be related to preadipocyte differentiation.

The importance of cholesterol in early stages of development could be the cause for the activation of pathways related to cholesterol metabolism and biosynthesis in both genetic types. For example, the *LXR/RXR activation pathway*, involved in the regulation of lipid metabolism, inflammation and cholesterol to bile acid catabolism, was enriched in both IB and IBxDU newborns ($p = 0.006$ and $p < 0.001$, respectively). On the contrary, at 28 days of age, this pathway was enriched in IB pigs [14]. The *atherosclerosis signaling pathway* (enriched in IBxDU pigs) or the *Cholesterol Biosynthesis* and the *Epoxysqualene Biosynthesis pathways* (enriched in IB pigs) were also identified as relevant pathways. However, phenotypic observations reflect a more active cholesterol and triglycerides biosynthesis in IB than in IBxDU piglets (Table 1). This could be due to the upregulation of different genes in each genetic type: upregulated genes in IB pigs associated with these pathways were closely related to the cholesterol and lipid metabolism (*FDFT*, *TF* and *APOM*). Accordingly, genes related to cholesterol and lipid metabolism such as *RXRG*, *USF1*, *LPL* or some apolipoproteins have been proposed as candidate genes involved in the familial combined hyperlipidemia in human [56, 90, 91], characterized by high levels of plasma cholesterol and triglycerides, as observed in IB pigs. Moreover, the aforementioned upregulated genes in IB pigs were connected in a network associated with functions such as *Amino Acid Metabolism*, *Molecular Transport* and *Small Molecule Biochemistry* (Fig 5). On the other hand, genes involved in such cholesterol-related pathways that were upregulated in IBxDU piglets were associated to the immune response (*MSR1* and *CCL2*).

The immune system is not fully developed at birth in pigs [92], and thus, related pathways and functions were upregulated in both in IB and IBxDU newborns. Some of these pathways include *PI3K Signaling in B Lymphocytes*, *April Mediated Signaling* or *B Cell Activating Factor Signaling* (enriched in IB pigs) and *Agranulocyte Adhesion and Diapedesis* or *IL-12 Signaling and Production in Macrophages* (enriched in IBxDU pigs). Moreover, the pathway *IL-17A Signaling in Fibroblasts* was enriched in both genetic types. On the other hand, the GO term *Positive regulation of immune system process* was enriched in IBxDU but not in IB piglets. This is consistent with the aforementioned upregulation of genes such as *MARCO* and *CXCL13* in Duroc-crossbred pigs, suggesting a more developed immune system in IBxDU than IB newborns. It has been previously reported that the immune system is affected by pig breed [93] and domestication [94]. An overrepresentation of genes related to the immune system was reported in wild boars when compared to domestic pig breeds [95]. However, to our knowledge there are no previous studies assessing differences in immune efficiency introduced by sire line.

Immune system-related genes are also involved in numerous other biological processes, such as fat accumulation, closely associated with inflammation [96, 97]. Thus, the enrichment of pathways related to the immune system could be related to lipid accumulation, although it has also been related to lower backfat thickness in pigs [98]. It is noteworthy that no biological functions related to the immune system were found enriched in IB pigs, which might suggest that upregulation of immune system-related pathways might be related to adipose tissue development rather than to the immune system development in IB pigs.

The reported active biological functions in IB and IBxDU pigs suggest a different predisposition for cell and tissue growth between genetic types, being IB pig metabolism intended to energy storage and IBxDU pig metabolism to cell growth and differentiation. These differences

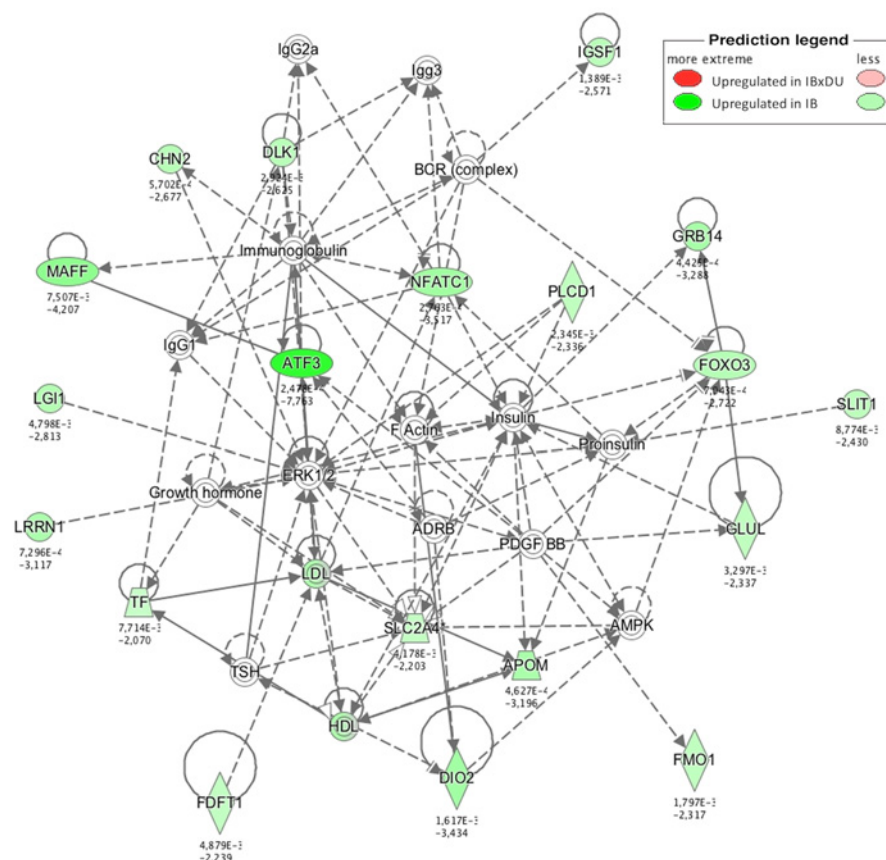


Fig 5. Gene network containing genes upregulated in Iberian (IB) pigs related to Amino Acid Metabolism, Molecular Transport and Small Molecule Biochemistry. The gene network shows interactions between cholesterol and lipid metabolism-related genes (LDL, APOM, FDF1, SLC2A4, etc.) upregulated in Iberian pigs.

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in biological regulation are in agreement with phenotypic differences found between the two genetic types.

Regulatory transcription factors

We performed a regulatory factors study to investigate the driving molecular mechanisms responsible for the differences in gene expression observed between genetic types.

A total of 723 TRF, obtained from the animal TFDB, showed expression values above 0.5 FPKM in our pigs and were thus considered as expressing TRF. Among them, 92 TRF potentially affected the gene expression profile in IB and IBxDU muscles (Table 4). We considered TRF that were either DE (7 TRF), identified by IPA software as regulators (45 TRF) or identified in the RIFs study (48 TRF) (Table 4).

In the present study, we found 7 TRF showing differences in expression level between genetic types. Most of them (*ABRA*, *ATF3*, *FOS*, *FOXO3*, *NOR1*, *TRIM63*) were upregulated in IB. These genes are related to muscle and adipose tissue development (Fig 1) and most of them have been mentioned in the previous section, but some of them may be highlighted and deserve additional comments. For example, one of the genes with greater expression differences (*ATF3*) was also an identified regulator in the *Animal TFDB*, suggesting an important role in the gene expression differences observed. *NOR1* showed also an important expression difference (6.56x) and codes for a nuclear receptor involved in a wide array of functions such as

Table 4. Potential regulators affecting gene expression that are: a) differentially expressed (DE) between IB and IBxDU; b) identified by Ingenuity Pathways Analysis (IPA) software or c) identified by RIFs study.

Ensembl ID	GENE	DE regulators		IPA—Regulators		RIFs study	
		<i>p</i> -value	FC Change	Z-score	<i>p</i> -value	RIF1(z)	RIF2(z)
ENSSSCG00000006036	<i>ABRA</i>	2.27E-06	-4.37				
ENSSSCG00000008123	<i>ARID5A</i>						2.01
ENSSSCG00000015972	<i>ATF2</i>				4.40E-02		
ENSSSCG00000015595	<i>ATF3</i>	2.48E-04	-7.76				
ENSSSCG00000000084	<i>ATF4</i>				1.42E-02		
ENSSSCG00000012241	<i>BCOR</i>				4.17E-03		
ENSSSCG00000013397	<i>BMAL1</i>						-2.22
ENSSSCG00000008377	<i>CCT4</i>						-2.11
ENSSSCG00000002866	<i>CEBPA</i>			-2.426	7.32E-03		
ENSSSCG00000002867	<i>CEBPG</i>					3.60	
ENSSSCG00000006752	<i>CSDE1</i>					2.45	
ENSSSCG00000011274	<i>CTNNB1</i>			-2.183	1.01E-02		
ENSSSCG00000014336	<i>EGR1</i>			-0.254	5.28E-03		
ENSSSCG00000010224	<i>EGR2</i>				2.01E-02		2.21
ENSSSCG00000008443	<i>EPAS1</i>				4.01E-02		
ENSSSCG00000002383	<i>FOS</i>	1.38E-03	-4.39	0.381	6.14E-03		
ENSSSCG00000012967	<i>FOSL1</i>				2.76E-02		
ENSSSCG00000007576	<i>FOXK1</i>					2.35	
ENSSSCG00000009370	<i>FOXO1</i>				1.03E-02	3.22	
ENSSSCG00000004387	<i>FOXO3</i>	7.04E-04	-2.72	-2.364	5.05E-06		
ENSSSCG00000001619	<i>FOXP4</i>					2.79	
ENSSSCG000000015733	<i>GLI2</i>					2.60	
ENSSSCG00000007720	<i>GTF2IRD1</i>				1.66E-02		
ENSSSCG00000000846	<i>HCFC2</i>					2.34	
ENSSSCG00000014388	<i>HDAC3</i>				2.36E-02		
ENSSSCG00000010472	<i>HHEX</i>				4.89E-02		
ENSSSCG00000004138	<i>HIVEP2</i>				1.70E-04		
ENSSSCG00000009327	<i>HMGB1</i>				3.67E-03		
ENSSSCG00000009704	<i>HMGB2</i>					2.55	
ENSSSCG00000008898	<i>HOPX</i>				1.66E-02		
ENSSSCG00000015985	<i>HOXD3</i>					3.59	
ENSSSCG00000005917	<i>HSF1</i>				9.28E-05		
ENSSSCG00000004238	<i>HSF2</i>				6.06E-03		
ENSSSCG00000014277	<i>IRF1</i>				2.49E-02		2.19
ENSSSCG00000003178	<i>IRF3</i>				3.13E-02		
ENSSSCG00000012853	<i>IRF7</i>				3.08E-02		
ENSSSCG00000008119	<i>KCNIP3</i>					2.34	
ENSSSCG00000010928	<i>KDM5B</i>						-2.10
ENSSSCG00000006928	<i>LMO4</i>					2.64	
ENSSSCG00000004528	<i>MBD2</i>				2.44E-02		
ENSSSCG00000000552	<i>MED21</i>					2.35	
ENSSSCG00000005720	<i>MED27</i>					2.18	
ENSSSCG00000006482	<i>MEF2D</i>				6.93E-03		
ENSSSCG00000012534	<i>MORF4L2</i>						-2.11
ENSSSCG00000013507	<i>MPND</i>						2.18
ENSSSCG00000012114	<i>MSL3</i>						2.34
ENSSSCG00000017882	<i>MYBBP1A</i>				2.07E-02		
ENSSSCG00000013375	<i>MYOD1</i>				7.26E-03		
ENSSSCG00000015475	<i>MYOG</i>				8.17E-06		
ENSSSCG00000010399	<i>NCOA4</i>					3.59	
ENSSSCG00000015987	<i>NFE2L2</i>			-2.206	2.44E-02		

(Continued)

Table 4. (Continued)

Ensembl ID	GEN	DE regulators		IPA—Regulators		RIFs study	
		p-value	FC	Z-score	p-value	RIF1(z)	RIF2(z)
ENSSSCG00000001952	<i>NFKBIA</i>			-1.279	3.40E-02		
ENSSSCG00000001703	<i>NFKBIE</i>				4.09E-02		
ENSSSCG00000005385	<i>NOR-1</i>	8.17E-04	-6.56				
ENSSSCG00000009856	<i>NOS1</i>	9.85E-03	2.29		3.78E-05		
ENSSSCG00000006689	<i>PIAS3</i>				3.69E-02		
ENSSSCG000000014437	<i>PPARGC1B</i>				1.65E-02		
ENSSSCG000000009746	<i>RAN</i>						-2.25
ENSSSCG000000009401	<i>RB1</i>			1.172	2.29E-02		
ENSSSCG000000008388	<i>REL</i>				2.04E-03		
ENSSSCG000000012981	<i>RELA</i>			0.749	6.16E-04		
ENSSSCG000000017071	<i>SAP30L</i>						-2.28
ENSSSCG000000011201	<i>SATB1</i>						-2.32
ENSSSCG000000001880	<i>SIN3A</i>						-2.12
ENSSSCG000000004952	<i>SMAD2</i>						2.43
ENSSSCG000000004524	<i>SMAD4</i>				4.94E-02		
ENSSSCG000000005232	<i>SMARCA2</i>						-2.47
ENSSSCG000000013629	<i>SMARCA4</i>				4.00E-02		
ENSSSCG000000006256	<i>SOX17</i>				3.29E-02		
ENSSSCG000000017403	<i>STAT3</i>				3.52E-02		
ENSSSCG000000017406	<i>STAT5B</i>					2.22	
ENSSSCG000000010638	<i>TCF7L2</i>				2.68E-02		
ENSSSCG000000001092	<i>TDP2</i>					2.44	
ENSSSCG000000001544	<i>TEAD3</i>						-2.06
ENSSSCG000000017950	<i>TP53</i>			-1.444	1.21E-04	3.48	1.69
ENSSSCG000000007208	<i>TRIB3</i>					2.84	
ENSSSCG0000000027684	<i>TRIM63</i>	7.02E-04	-2.46		1.25E-02		
ENSSSCG000000015370	<i>TWIST1</i>				3.28E-03		
ENSSSCG000000006790	<i>WDR77</i>						-2.18
ENSSSCG0000000011953	<i>ZBTB11</i>					2.10	
ENSSSCG000000007958	<i>ZNF174</i>					2.31	
ENSSSCG000000003233	<i>ZNF175</i>					2.86	
ENSSSCG000000002838	<i>ZNF423</i>					2.26	
ENSSSCG000000003070	<i>ZNF45</i>					2.24	
ENSSSCG000000007767	<i>ZNF668</i>						2.26
ENSSSCG000000001206	<i>ZSCAN26</i>						-2.84
ENSSSCG000000002877	<i>ZNF181</i>					2.50	
ENSSSCG000000003244							2.05
ENSSSCG000000011620							-2.41
ENSSSCG000000011943							-2.07
ENSSSCG000000013384							1.96
ENSSSCG000000016700						3.43	

FC: Fold-Change

Z-score: reflects the activation state of predicted transcriptional regulators. It is based on the experimentally observed gene expression, and on literature-derived regulation direction information, which can be either “activating” or “inhibiting”

RIF1 (z) extreme scores identify those transcription factors that are consistently most differentially co-expressed with highly abundant and highly DE genes. Bootstrap 95% and 99% confidence intervals for RIF1 z-scores: -1.996/2.074 and -2.883/2.918, respectively.

RIF2 (z) extreme scores identify transcription factors with the most altered ability to predict the abundance of DE genes. Bootstrap 95% and 99% confidence intervals for RIF2 z-scores: -2.036/1.953 and -2.609/2.490, respectively

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inflammation, cell cycle regulation, apoptosis, steroidogenesis, adipogenesis, angiogenesis and energy metabolism. Moreover, it has been found overexpressed in obese when compared to normal humans and its expression went back to normal values after fat loss [99]. These findings are in accordance with the results of the present study, where animals with higher IMF content showed greater muscular expression of *NOR1*.

IPA software is a potent tool to identify regulators based on previous knowledge and bibliographic references. On the other hand, the RIFs analysis, based on co-expression information in our dataset, complements the bibliographic approach (IPA). The combination of these two methods is a powerful strategy to identify TRF. In the present work, IPA software identified 45 TRF affecting the DE genes, while the RIFs metrics identified 48. Four TRFs were identified following the two approaches (*EGR2*, *FOXO1*, *IRF1* and *TP53*). *EGR2* is a growth factor that promotes adipocyte differentiation [100], while *TP53* has been reported to affect cell metabolism [101, 102]. *FOXO1* is a member of the forkhead family of TRF, which exerts important regulatory functions in developmental processes including muscle development [103, 104]. *FOXO1* regulates expression of several adipogenic genes, including *PPARG* [105] and muscle cells differentiation in association with *SMAD* [106], although inconsistent information exists regarding its specific role [106, 107]. Moreover, several TRF with known functions on adipogenesis and lipid metabolism were identified in the present regulators analysis (*CEBPA*, *CEBPG*, *ZFP423*, *EGR1*, *ATF2*, *ATF4* and *PPARGC1B*). *PPARGC1B* is involved in fat oxidation, non-oxidative glucose metabolism, mitochondrial biogenesis and the regulation of the energy expenditure [108] and was also identified as a potential regulator of gene expression differences at 28 days of age [14]. The identification of these genes as regulators in the present study suggests its implications in adipogenesis and in the observed fatness differences associated to sire breed.

Regulation of myogenesis is also a very complex process and several TRF involved on it have been identified (*FOXK1*, *FOXO4*, *MEF2D*, *MYOD1*, *HDAC3* and *MYOG*). These genes play a central role in myogenesis regulation, acting sequentially in a signaling chain [29, 109–113]. Due to the importance of muscle development in the pork industry, several studies have assessed the differential expression of myogenic regulatory factors between breeds with different muscle growth potential [20, 29, 83, 114]. The identification of these myogenic regulators as TRF in the present study, suggests that myogenesis is also activated immediately after birth, although it has been reported that expression significantly decreases in pigs after 90 days post-conception [114]. However, muscle development timing remains unclear, since it has also been hypothesized that muscle is still under development in 3 months-old Casertana pigs [83]. Moreover, genes involved in myogenesis inhibition (i.e. *SMAD* and *TWIST1*) were also identified as TRF affecting gene expression in IB and IBxDU pigs. The presence of both myogenesis activating and inhibiting genes reflects the complex regulation of this process in newborn piglets.

Muscle deposition not only depends on myogenesis; regulation of process such as angiogenesis, or protein degradation is also decisive in final muscle deposition. *SOX17* and the heat shock transcription factor family members *HSF1* and *HSF2*, involved in such processes [115–117], were identified in this TRF study.

To better understand interactions between the identified DE genes and TRF, a pathways enrichment analysis was performed combining information obtained in both analyses. The adipogenesis pathway was the most enriched pathway (S5 Table), accordingly with the differences in fatness observed in BF muscle of IB and IBxDU piglets. As observed in Fig 6, the adipogenesis pathway includes several TRF (*ZFP423*, *P53*, *FOXO1*, *STAT5* or *EGR2*) and DE genes (*SLC4A2/GLUT4* and *DLK1*). It is noteworthy that *PPARG*, considered as the master regulator of adipogenesis, was not found to be DE or to regulate gene expression in newborn IB and IBxDU pigs. However, 6 out of the 8 identified TRFs involved in this pathway, regulated directly or indirectly the expression of *PPARG*.

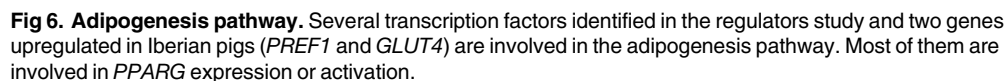
Structural analysis from RNA-Seq sequence data

A total of 433,667 putative variants were identified in IB and 461,438 in IBxDU pigs.

The total numbers of polymorphisms meeting the filtering criteria, and the location, frequency, amino acid change and variant type distribution in both genetic types are shown in [Table 5](#). Both genetic types showed a similar variant number, with similar location and variant type distributions. Regarding variant frequency distribution, IB group showed slightly higher number of potentially fixed variants (frequency equal or higher than 90%) while crossbreds showed higher number of segregating variants (frequency lower than 90%), in agreement with the expectations for the comparison of one pure line with high inbreeding level as the Iberian pig [23] and an F1 cross. Nevertheless, frequency estimations have to be considered with caution due to the limited sample size. Variants at different allelic frequencies in both genetic types were of special interest, especially those potentially fixed in IB and segregating in IBxDU (7,741 variants). Moreover, variations in protein-coding regions may give rise to non-synonymous changes in the amino acid sequence of the encoded protein and thus, are more likely to affect the protein structure and function [118] probably leading to critical effects on a phenotype of interest [119]. Out of the 7,741 potentially relevant variants, 846 produced a non-synonymous change.

One of the goals of the present study was to identify structural variants in candidate genes involved in differences between IB and IBxDU piglets. Candidate genes were selected based on the following criteria: TRF simultaneously identified following IPA and RIF approaches (*EGR2*, *FOS*, *FOXO1*, *FOXO3*, *IRF1*, *NOS1*, *TRIM63*, *ABRA*, *ATF3* and *TP53*) and those coinciding with TRF previously identified in an IB and IBxDU piglets transcriptome comparison at 28 days of age (*HOXA9*, *STAT5B*, *ATF4* and *PPARGC1B*).

In order to identify structural variants in these strong candidate genes, we looked for differentially segregating SNPs in IB and IBxDU pigs. The total variant number in the whole set of selected genes was slightly larger in IBxDU than in IB pigs (365 and 294, respectively). Out of the identified variants, 177 were present in both genetic types, 117 were present uniquely in IB and 188 in IBxDU piglets. The supplementary file 6 ([S6 Table](#)) shows all the variants found in the 14 genes of interest. The genes *ATF4* and *HOXA9* did not show any polymorphism. The genes *ATF3*, *STAT5* and *ABRA* showed common polymorphisms between the two genetic types that were fixed or in high allelic frequency in IB pigs. Among them, *ABRA* presented 11 variants fixed in IB that segregate in IBxDU. None of these polymorphisms led to amino acid changes, nevertheless they might affect mRNA processing (splicing, maturation, stability, transport) and translation and thus, lead to altered mRNA folding and even expression [120]. Moreover, the 11 variants were found in cosegregation, which might lead to relevant changes in the mRNA. The identified forkhead family TRFs (*FOXO1* and *FOXO3A*) showed fixed and high frequency variants uniquely present in IBxDU pigs. Nevertheless, we identified a low frequency variant in IB pigs that produced a non-synonymous amino acid change in *FOXO1* gene. *NOS1* and *TP53* genes presented several variants in both genetic types. Variants in *NOS1* gene were mainly associated to the IBxDU type, two of them causing amino acid changes. On the other hand, variations in *TP53* were observed generally in IBxDU pigs. Considered as a tumor suppression gene, TP53 protein responds to diverse cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. It has been recently reported as a novel candidate for muscle development in pigs [121] and was also involved in the adipogenesis pathway ([Fig 6](#)). The *PPARGC1B* and *TRIM63* genes were the most polymorphic ones among the studied genes (89 and 94 variants, respectively). Some of the variants found in these genes were associated to amino acid change. One of the variants identified in the *PPARGC1B* gene, at medium frequency in IB and



absent in IBxDU, was predicted by SIFT to produce a deleterious amino acid change. Also a GCA-deletion, fixed in IB and segregating in IBxDU pigs, produces the lack of an alanine residue in the coded PPARGC1B protein. On the other hand, 15 variants raised missense amino acid changes and 7 produced frameshift alteration in the *TRIM63* gene, which maintains muscle protein homeostasis by tagging the sarcomere proteins with ubiquitin for subsequent degradation by the UPS [122]. Moreover the polymorphism *ENSSSCT00000028044:c.163G>C* was predicted to be deleterious by SIFT metrics. Thus, these changes might potentially cause altered

Table 5. Number of sequence variants present in IB and IBxDU pigs RNA-Seq data and its distribution according to localization, frequency and polymorphism type.

	IB	IBxDU
Total number of variants (after filtering)	120,998	125,382
Variant localization		
UTR Region	20,683	21,808
Coding region	100,315	103,574
Potentially fixed and segregating variants		
Fixed ($\gamma \geq 90\%$)	46336	40454
Segregate ($\gamma < 90\%$)	74662	84928
AA change	18092	18556
Variant type		
SNV	88,415	92,416
MNV	11,842	11,873
Insertion	7,863	7,987
Deletion	9,539	9,699
Replacement	3,339	3,407

IB: Iberian purebred piglets

IBxDU: Duroc crossbred Iberian piglets

Filtering: Variants with coverage < 30 or frequency < 5% were dismissed

UTR Region: Untranslated region

AA change: Variants causing a aminoacid change in the protein

SNV: Single nucleotide variant

MNV: Multiple nucleotide variant

Replacement: Neighboring SNVs and insertions or deletions.

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function in the proteins. Previous studies associated polymorphisms in *PPARGC1B* gene with type 2 diabetes [123] and subcutaneous adiposity [124] in human. Moreover, *PPARGC1A* codes for a homologous protein of *PPARGC1B* and has been associated in pigs to carcass composition traits such as leaf fat weight, backfat thickness, and belly weight in a Meishan cross population [125].

Variants showing different segregation between genetic types may be associated with functional genetic differences, which potentially could affect gene regulation and metabolism. Specially, the polymorphisms associated to aminoacid changes detected in *FOXO1*, *NOR-1* and *PPARGC1B* genes might be candidate polymorphisms to partially explain the differential muscle and adipose growth between IB and IBxDU.

Conclusions

In the present study, differences in phenotype, transcriptome, metabolic pathways and transcriptional regulation were found between BF muscles from purebred and Duroc-crossbred Iberian newborn pigs. Phenotypic differences regarding body size, plasma cholesterol levels and IMF content were remarkable, even at this early age. This is concordant with the transcriptomic study, which revealed several DE genes related to adipose and muscle tissues development such as *DLK1*, *FGF21* and *UBC* genes. The interpretation of these results pointed out a differential regulation of several biological processes. For example, lipid metabolism and muscle atrophy were upregulated in IB pigs, in accordance with the greater adipose accretion and lower muscle growth observed in Iberian pig breed. However, muscle growth was upregulated

in IBxDU pigs, characterized for a higher muscle development than IB pigs. These processes are closely related to meat quality and production traits. Protein catabolism and cholesterol metabolism were enriched in both genetic types, although phenotypic differences in plasma cholesterol suggest greater activation of this process in IB pigs. These results contribute to the understanding of molecular mechanisms driving phenotypic differences observed in IB and IBxDU pigs. Moreover, findings regarding lipid metabolism regulation might be of interest in research related to metabolic alterations in other species such as humans.

Also, we identified TRF that potentially regulate the observed transcriptomic differences. The different employed approaches allow highlighting several TRF especially interesting such as *CEBPs*, *EGRs*, *ATFs*, *PPARGC1B*, *FOXOs*, *TRIM63*, *MEFD2D*, *MYOD1* or *MYOG*.

Finally, genetic structural variations that might be associated to changes in expression and protein function were identified. Differentially segregating SNPs in IB and IBxDU piglets associated to those TRF that were more consistently identified were of special interest. Among them, *PPARGC1B* and *TRIM63* showed non-synonymous variants that might differentially regulate their function in IB and IBxDU pigs. Taken together, results found in the present study provide information about candidate genes and genetic polymorphisms potentially involved in phenotypic differences between IB and IBxDU associated to meat quality and production traits. Further validation of these genes and polymorphisms would contribute to their use in future breeding programs.

Supporting Information

S1 Table. Gene information, primer sequences, amplicon size and efficiency of genes selected for qPCR validation.

(XLSX)

S2 Table. Genes found differentially expressed between purebred (IB) and Duroc-crossbred (IBxDU) newborn pigs.

(XLSX)

S3 Table. RNA-Seq and qPCR validation results and correlation coefficient (r) between the two used methodologies.

(XLSX)

S4 Table. Enriched biological functions identified by IPA software in purebred (IB) and Duroc-crossbred (IBxDU) Iberian pigs ($p < 0.01$).

(XLSX)

S5 Table. Canonical pathways enriched in IB and IBxDU animals based on DE genes and TFR identified either by IPA or by RIFs study.

(XLSX)

S6 Table. Structural variants identified in 14 candidate genes (*EGR2*, *FOS*, *FOXO1*, *FOXO3*, *IRF1*, *NOS1*, *TRIM63*, *ABRA*, *ATF3*, *TP53*, *HOXA9*, *STAT5B*, *ATF4* and *PPARGC1B*).

(XLSX)

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Author Contributions

Conceived and designed the experiments: CO CLB AGB. Performed the experiments: MA BI AIR RB YN CB. Analyzed the data: MA AF AIF CO JFM AC. Contributed reagents/materials/analysis tools: AGB BI AIR. Wrote the paper: MA CO.

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